Thymidylate Synthase Inhibition: A Structure-Based Rationale for Drug Design

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Abstract: Thymidylate synthase (TS) is a very interesting target in antiproliferative diseases. Its inhibition causes thimineless death of the cells and compounds inhibiting TS are widely used in anticancer therapy. The classical antifolate TS inhibitors are structural analogs of the folate cofactor; they often share the same metabolic pathways and this causes the development of resistance inside the cells. A detailed analysis of the available x-ray crystal structures of the complexes of the enzyme with different substrates and inhibitors support the finding of a structural basis of their biological activity. TS inhibitors nonstructural analog of folate, non-analog antifolate inhibitors (NAAI), are welcome as a new interesting research topic. Among the most recent and interesting ones, compounds from Agouron related to the indole structure, are independent on the folate metabolism, highly active and specific for human TS. Other compounds, phthalein derivatives, can inhibit TS enzymes from various sources and show an interesting biological activity profile: they inhibit better bacterial and fungal TS than human TS. The x-ray crystal structures of some of these inhibitors with TS show that they bind in a different binding site from that of the classical folate TS inhibitors. This indicates a potential allosteric binding site useful for future drug discovery studies. © 1998 John Wiley & Sons, Inc. *Med Res Rev*, **18**, No. 1, 21–42, 1998.

Key words: thymidylate synthase; inhibition; structure-based design; antifolates; NAAI

I. OVERVIEW

Thymidylate Synthase (TS) is a macromolecular protein that catalyzes the reductive methylation of deoxyuridine -5′-monophosphate (dUMP) to thymidine-5′-monophosphate (dTMP) which is then phosphorylated to thymidine-5′-diphosphate (dTDP) and thymidine-5′-triphosphate (dTTP) and finally incorporated into DNA, being a substrate of DNA polymerase (Fig. 1).

This is a key step in DNA biosynthesis, the only *de novo* pathway to dTMP synthesis.[1–3] TS is present in almost all living organisms including bacteria, DNA viruses, and protozoa, and is one of the most conserved enzyme known in the evolutionary scale.[4] It has always been considered a key target in anticancer chemotherapy, and traditional Medicinal Chemistry has come up with some antimetabolites that interfere with dUMP and folate cofactor (MTHF) functions.

One of the best-known and widely used anticancer drugs is 5-fluorouracil (FU).[5] It is a prodrug that can be deoxyrybosylated and phosphorylated and acts as a mechanism-based inhibitor. Of the antifolates, compound CB3717, a N5,N10-propargyl-quinazoline derivative of dideazamethotrex-

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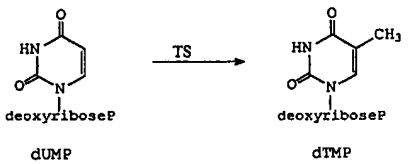


Figure 1. TS catalyzed reaction.

ate, is historically the first important TS inhibitor. Several antifolates structurally related to the folate cofactor are currently in clinical trials but are not yet used in therapy. The main problems with these compounds are: low selectivity, multiple targets of action, high toxicity, and development of resistance. They cannot discriminate between normal TS and tumor TS; moreover, both antifolates and dUMP analog inhibitors have many loci of action inside the cells.[6] Many studies have been carried out on TS inhibition.[7–9] The mechanism of action has long been studied, and in recent years mutagenesis studies have produced interesting results regarding the structure-function relationship. Even though great progress has been made in this field, there has been no breakthrough in the relationship between Biochemistry and Medicinal Chemistry.

Owing to the recognition of TS as a target in many infectious diseases, and to the recent understanding of the regulatory function of TS on some oncogenes of mRNA, there has been a renewal of interest in this field.

II. TS FUNCTIONS

TS is a two-substrate enzyme that accepts both dUMP and tetrahydrofolate to form a ternary complex through which reductive methylation of dUMP to dTMP occurs. dTMP is then phosphorylated to dTTP in the reactions catalyzed by cellular kinases, and in this form is a substrate of DNA polymerases and is incorporated in the DNA. This reaction provides the *de novo* cellular thymidylate nucleotides necessary for DNA synthesis and repair. TS inhibition results in a thymineless state, which is toxic to actively dividing cells.

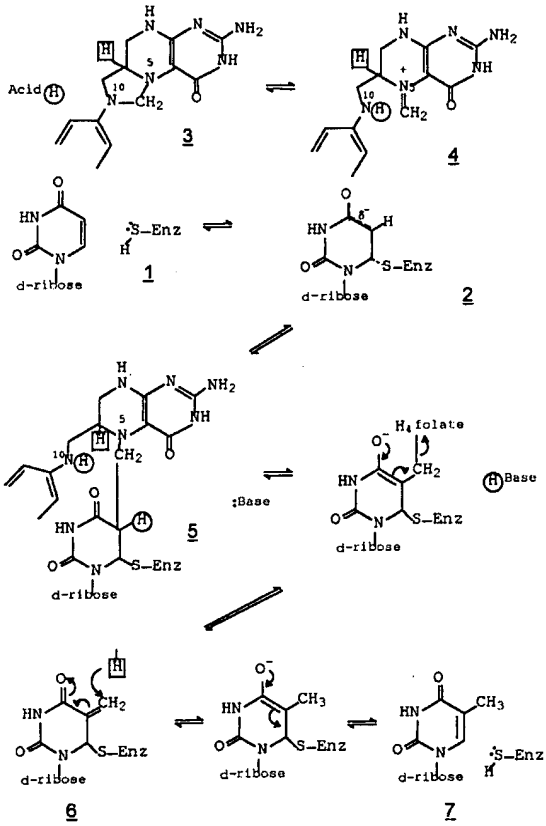
This cytotoxicity is probably effected by DNA fragmentation resulting from dTTP depletion, which increases misincorporation of 2′-deoxyuridine-5′-triphosphate (dUTP). Cells containing the salvage enzyme thymidine kinase, which uses circulating thymidine, can circumvent this cytotoxicity. However, in normal tissue and in some tumor cells, the circulating thymidine concentration may not be sufficient to achieve a significant effect.[10]

TS can influence the 2′-deoxyadenosine-5′-triphosphate/ thymidine triphosphate ratio inside the cell, thus indirectly modifying the incorporation in DNA of the component bases.[11] The catalytic reaction is described in Scheme 1.[3]

TS 1 reacts with dUMP to give the noncovalent binary complex 2, which rapidly binds the cofactor (4), to give the noncovalent ternary complex (TS-dUMP-cofactor). The protonation of the cofactor in position 10 changes the inactive form 3 to the active form 4 in which MTHF is in the protonated iminium form. The noncovalent ternary complex changes into the covalent ternary complex 5, which is an unstable intermediate.

When the catalytic thiol (Cys-198)* of TS gives a nucleophilic attack on C6 of the uracil ring of dUMP, a Michael-type adduct is formed and the carbon unit of the cofactor gives an electrophilic at-

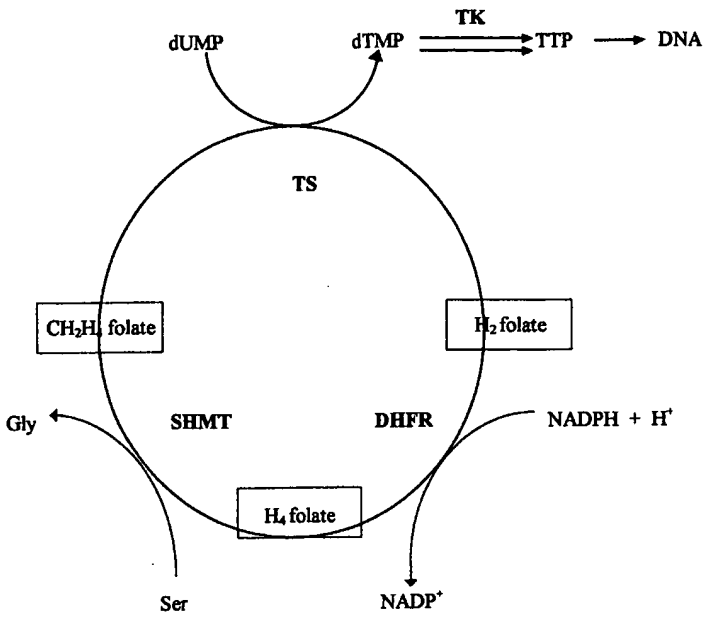
*The numbers are referred to *Lactobacillus casei* thymidylate synthase, unless otherwise stated.



Scheme 1. Mechanism of action of Thymidylate synthase.

tack on C5 of dUMP trans with respect to Cys-198. The hydrogen at C5 of the uracil ring is abstracted by an unidentified base in the receptor site. The methylene group is reduced to a methyl group through hydride transfer from C6 of the cofactor (6), which is oxidized at the N5—C6 bond; at the same time, the thiol group undergoes β-elimination to reinstate the double bond and afford the reaction product, dTMP.

TS enters the thymidylate cycle that is necessary to return the oxidized folate to the reduced form (Fig. 2).[1] Two other enzymes are part of this cycle: dihydrofolate reductase (DHFR) and serine-hydroxymethyl transferase (SHMT). The former can catalyze the reduction of dihydrofolate to tetrahydrofolate, the latter can catalyze the methylene transfer from serine to the reduced cofactor. TS has an autoregulatory function. In fact it can interact with those genes on mRNA that promote its own expression.[12–14] This tendency to interact with mRNA is expressed through its regulatory function on some oncogenes of mRNA. This latter role is not well understood and still being studied. TS can interact with cancer-related genes including p53, a tumor supressor gene and bcl-2 proto oncogene. The bcl-2 gene is usually expressed in a wide variety of tissues in which a normal continuous cellular turnover is present. When this gene is blocked, the cell dies as it approaches apoptosis. The p53 gene can block the cell cycle at the G1 phase, when a DNA repair process is under way. Consequently the cell has time to finish the repair of the damaged DNA before the cell di-



TS= Thymidylate Synthase

SHMT= Serin Hydroxymethyl Transferase

DHFR= Dhydrofolate Reductase

TK= Thymidine Kinase

H_2 folate= Dhydrofolate

H_4 folate= Tetrehydrofolate

C_1H_4 folato= N^5,N^{10}-Methylentetrahydrofolate

Figure 2. Thymidylate synthase cycle.

vision. If the repair process fails, the cell proceeds towards apoptosis and is eliminated. If p53 is blocked or mutated, no salvage pathway can work and a tumoral phenotype can be generated. TS can interact with both p53 and bcl-2 genes, especially if it is present in high concentration, as in the tumor tissues. In fact, it seems that TS can block the p53 gene specifically, and as a consequence, tumor generation is favored. High levels of TS are also present in drug-resistant cells, which leads to highly complicated metabolic situations. The normal or abnormal expression of a certain gene can affect the ultimate response of tumor cytotoxic drugs. *In vitro* translation of p53 mRNA was specifically repressed with the addition of exogenous human TS in human colon cancer H630 cells, and TS was shown to complex with p53 and c-myc mRNAs. The role of this mRNA protein interaction, although not yet clear, suggests that TS may play a regulatory role in p53 and c-myc cellular expression. Moreover, the c-myc protein decreased while the p53 and Rb proteins increased after the exposure of HCT-8 cells to ZD1694 (antifolate TS inhibitor). Although preliminary, these data are interesting because such interactions may play a critical role in retarding cell progression through the S-phase and initiating apoptosis after drug exposure. If the cytotoxic activity of TS inhibitors is mediated ultimately by the induction of apoptosis, then mutations in the genes encoding these proteins or interference with their activity could decrease the expected cytotoxicity. Specific

sequences of the protein structure in human TS seem to be involved in this interaction with mRNA.[15] Preliminary reports from studies of p53 function in patients with advanced colorectal cancers suggest that patients with point mutations in p53 may not respond to chemotherapy with FU and leucovirin (LV).

One more aspect of the TS function is its involvement in the replitase complex. The enzymes of DNA polymerization and DNA precursor synthesis are assembled in the replitase complex during the S-phase of the cell cycle. The presence of this multienzyme complex, containing the enzymes of both DNA precursor biosynthesis and DNA replication, is widely reported in mammalian cells. *In vivo* catalytic activity of the enzymes, such as TS and DNA polymerase, are confined to the S-phase, and this activation of the enzymes during this phase suggest that they are associated with the replitase complex.[16] It has been shown that a variety of antimetabolites cross-inhibit TS *in vivo,* but not *in vitro.* For instance, hydroxyurea (HU) inhibits ribonucleotide reductase and also TS *in vivo.* On the other hand, this HU cannot inhibit the isolated TS enzyme. Several mechanisms have been suggested as being implicated in this inhibition but the most convincing foresees cross-inhibition occurring by allosteric interactions within the structure of the replitase complex.[17] The mechanism of inhibition in the multienzyme complex is of interest, since it suggests a new mode of inhibition of Human TS (HTS) through some conformational change occurring in the quaternary structure, as already suggested.[15]

III. ENZYME STRUCTURE AND FUNCTIONS

The TS enzyme is a homodimer of 74,000 daltons and consists of about 316 aminoacids. Primary sequences show that TS is one of the most highly conserved enzymes known.[18] The primary structures of about 30 TS enzymes are known, including those of humans, bacteriophages, and plants. Some of them have been cloned and are readily available[19] for functional and inhibition studies. The primary structure is highly conserved: approximately 27 amino acids are completely conserved, and 165 (80%) are conserved in more than 60% of the organisms. In particular, the topological active site of dUMP is formed by 32 amino acids, of which 16 are conserved.[4]

X-ray crystal structures of TS from several prokaryotic species, e.g., *Escherichia coli,*[18] eukaryots, such as *Lactobacillus casei,*[20,21] *Leishmania major,*[22] and *Pneumocistis carinii,*[23] as well as from T4 phage,[24] have been determined and indicate that the tertiary structure is well conserved. The first mammalian TS structure has also recently been solved.[15] This latter structure is not highly refined and some sequences are very disordered, but it remains the only human TS structure available. Many crystal structures of TS complexed with substrates and ligands have also been determined.[25–29] They are very important for the understanding both of the mechanism and of inhibition[30–35] and gave rise to the structure-based approach that complemented the traditional methodology in medicinal chemistry. Knowledge of the 3D structure of the enzyme addressed the functional studies in a rational way. Mutagenesis studies have been developed through site-directed mutagenesis methods in order to define the functional role of each residue involved in the substrate binding.

These studies consist of selective modification of one or more amino acids in the active site.[19] The mutants are characterized through the kinetic parameters Km and kcat that are related to the physico-chemical properties of the substituted aminoacids, thereby facilitating a structure-function relationship study. The studies on mutagenesis demonstrated that many of the highly conserved residues can be changed without dramatically compromising enzymatic activity. This ability to tolerate aminoacid substitution is called *structural plasticity.* In particular the dUMP substrate active site has been closely studied. Figure 3 shows a detail of the x-ray crystal structure of the binary complex LcTS-dUMP.[21] The most important residues involved in dUMP binding are to our knowl-

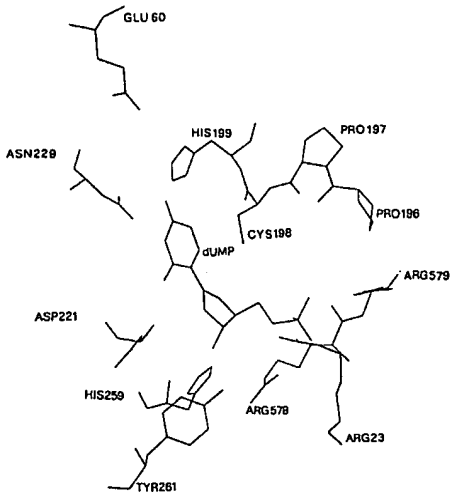


Figure 3. Details of the x-ray crystal structure of the binary complex LcTS-dUMP: dUMP binding site.

edge: Cys 198, Asn 229, Arg 178′ and Arg 179′, Glu 60 and Val 316. Cys 198 has a thiolate group that can give a nucleophilic attack on the uracil ring of the substrate. Every mutation at this residue inactivates the enzyme, since the covalent bond with the substrate is essential for the catalytic reaction. Asn 229 is part of a hydrogen bond network including N3—C4=0.

Mutagenesis studies have shown that the substitution of this amino acid causes the destruction of this hydrogen bond network and the decrease or disappearance of catalytic activity,[36–38] but it can still catalyze the methylation reaction of 3-methyl dUMP. If the substituted amino acid is Asp, there is a change in specificity: the enzyme is no longer a deoxyuridylate methylase, but a cytidylate methylase, which catalyzes the reaction with dCMP instead of dUMP. This amino acid would therefore appear to have a very important role in specificity.[30,39,40]

The two arginines (178′, 179′, reported as 578, 579 in Figure 3) belong to the opposite subunit and they bind to the phosphate group of the deoxyribose ring. Substitution with other amino acids depresses the catalytic activity.[41] Further studies have been carried out on the other amino acids of the active site.[42,43a,b] For instance Pro 196, Pro 197, and His 199[42] are highly conserved but their substitution with other amino acids is very well tolerated. For some time it was thought that His 199 played an important role in the hydrogen bond network, serving as the base abstracting the proton to form the enolate intermediate (Scheme 1), but recent findings indicate that this seems to involve a structural water molecule instead located close to the C4 carbonyl group. The study of the Val 316 C-terminal residue has been particularly important: spectroscopic studies (fluorescence,[44] UV-Vis,[45] circular dicroism,[46] and NMR[47]) have highlighted its involvement in the development of the catalytic event. It takes part in the conformational change occurring upon covalent binding of the cofactor to the binary complex TS-dUMP. This process makes for the optimum directionality of the components during the catalytic reaction. In fact, when the conformational change occurs the cofactor gives an electrophilic attack on the C5 of dUMP. The x-ray crystal structures of TS-folate-FdUMP ternary complex shows that in this latter complex the carboxy terminus residue is shifted far from its original position in the unbound form of about 4 /Å towards the dUMP active site as a gate. Of course, this movement causes the partial modification of the general hydrogen bond system that involves the amino acids of the active site.

Drastic deletion of the small domain portion of LcTS enzyme has also been performed[48] and the inactive enzyme reactivated after mutation, which converts LcTS into an active EcTS-like enzyme. This small domain (residues 90-139) is typical of some TS, such as LcTS and *Staphylococcus aureus* TS, but is absent in HTS, thus suggesting an important role in the specificity of the enzyme.[20,49] This all contributes to the correct understanding of the role of the amino acids of the enzyme involved in binding and catalysis and enables medicinal chemists specifically to target some amino acids essential for enzyme health and block them. While the dUMP active site has been closely studied, the folate binding site has not yet been thoroughly explored.

IV. ENZYME INHIBITORS

A. dUMP Analogs

The inhibition of thymidylate synthase has been accomplished by the classical method of designing antimetabolites capable of blocking TS activity by means of analogs of the substrate and of the folate cofactor. In the past many nucleotides have been synthesized bearing modifications of the uracil ring in position 5 and, to a lesser extent, 6.[1,2] The most important substituents are NO2, CF3, halogen, and vinyl[1,50] (Fig. 4).

Other modifications have been made to the carbonyl at C4, which can be substituted by an amino group (cytidine derivatives).[51] The best-known and most important compounds are the fluoropyrimidines and their nucleosides.[5,52] Of these, 5-fluorouracil is a prodrug because it is deoxyribosylated and then phosphorylated inside the cell. As a monophosphate it can bind to TS and acts as a suicide inhibitor. Depending on the substituent at C5, the mechanism of action can change, and the compounds can be mechanism-based inhibitors, as in the case of FdUMP, or acylating agents, as in the case of trifluoromethyl uracil.[53,54] The main problems with the nucleotide derivatives are their low selectivity and high toxicity. In fact, the nucleotidic structure makes these molecules good ligands of many biological macromolecules, as in the case of the enzymes involved in the metabolic pathway of the nucleosides and nucleotides or the nucleic acids. In the case of FdUMP, the most intensively studied and also used inhibitor as a probe for TS function studies, the x-ray crystal structure of the ternary complex of the compound with the folate analog (CB3717) is solved and a good superimposition of this structure with that of the ternary complex of TS-dUMP-CB3717 can be seen (Fig. 5).

The inhibitor can bind in the same site as dUMP does; the cofactor forms a covalent bond with the C5 moiety of the nucleotide. From the structural point of view, the competitive inhibition pattern of FdUMP and the covalent ternary complex formation can be explained. In fact the x-ray crystal structure of the ternary complex of FdUMP with EcTS and the folate inhibitor CB3717 shows that the nucleotide binds in the same binding site of dUMP (Fig. 5). Recently other x-ray complexes of nucleotide inhibitors have been solved, which provides a structural basis for the enzyme's kinetic behavior.[53] Of the most recent inhibitors the dihydrouracil derivatives[55] or pyridoxal phosphate derivatives,[56] sulphonamide derivatives,[57] and ethinyl derivatives[50,58] are of note (Fig. 4). More nucleosidic derivatives have been synthesized[59–63] but no interesting advances in either inhibitory potencies or pharmocokinetic properties have been made to date.

In general, no selectivity between tumor TS and normal cell TS has yet been established, and the driving force of the inhibition is the higher intracellular level of TS in hyperproliferative tissues, so that a higher inhibitor concentration can be achieved in tumor cells. Currently, there is great interest in TS inhibitors, and the most efficacious and recent compounds are antifolates inhibiting human TS and acting as anticancer drugs. Our interest is also mainly focused on this new type of inhibitors.

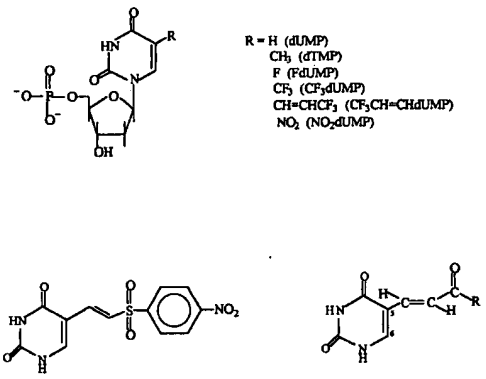


Figure 4. dUMP analogs inhibiting TS.

B. Antifolates

The antifolates active against DHFR, such as methotrexate (MTX), are able to interrupt the TS cycle because many of them can also inhibit TS, and many antifolate TS inhibitors can inhibit DHFR.[64] Until recently little attention has been paid to selectivity; it being supposed that broad spectrum compounds could be more efficacious (combination chemotherapy).[65] This concept has now been completely reversed, because selectivity must always be the top priority, and this is important at different levels:

(a) compounds with a folate structure can interfere with many biochemical steps involving folate metabolism,[66] so the toxic effects due to the interaction with other macromolecular targets of the metabolic pathway are enhanced;
(b) even though great progress has been made in the study of the enzyme functions, much remains to be discovered. We should therefore acknowledge the important contribution made by biological targets other than the one we are studying. For example, in the case of TS, its functional role is not only the methylation of dUMP to dTMP; it also regulates some genes

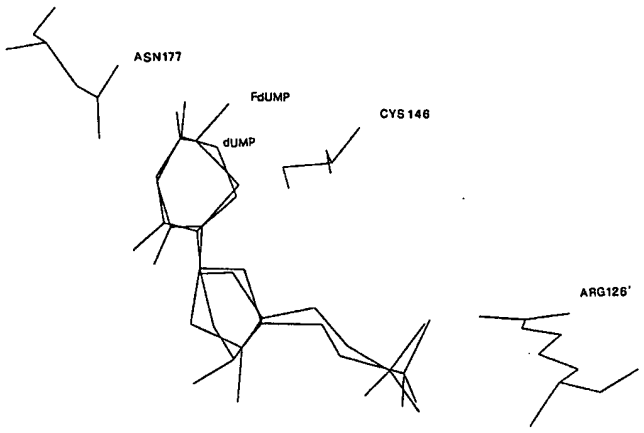


Figure 5. Rigid matching of the x-ray crystal structures of the ternary complex EcTS-FdUMP-CB3717 and EcTS-dUMP-CB3717: dUMP binding site.

by interacting with mRNA, and this must be given due weight. The above considerations are extremely important in targeting cancer cell TS in anticancer chemotherapy; they are not so important in antibacterial, antifungal or antiviral drug design, where the chief concern is to kill the infectious agent.

In general, the classical folate analogs, especially the most recent ones, are very potent TS tight-binding inhibitors. The preliminary biological screenings are in general very promising, but the antifolates usually turn out to be toxic. From the structural point of view, they are correlated with the folate structure. They have a highly lipophilic region, the pteridinic ring, which can be substituted by a quinoline or quinazoline ring, and a hydrophilic part formed by the *p*-amino-benzoate portion linked to the glutamic acid residue. The enzymatic counterpart has a complementary polarity: highly hydrophobic in the pterdine ring binding site and hydrophilic in the glutamic binding site, where many lysine residues are present. Many derivatives have been made by changing different parts of the molecule, and structure-activity relationships have been studied.[64]

In consideration of the many structural modifications that have been made over the past four decades with the aim of qualitatively or quantitatively altering the therapeutic spectrum of MTX and, more recently, of generating selective TS antifolate inhibitors, the molecule of MTX may be conveniently dissected into five regions, as indicated in Figure 6.

Region **A** includes C for N substitution at position 1 or 3, replacement of the amino group at position 4 by a carbonyl group and of the amino group at position 2 by a methyl group. Region **B,** comprising the pyrazine moiety, has proved an important field for exploration where C for N substitution generates the 5,8 dideaza analog, whose lead is CB3717.

Region **C** is usually referred to as the bridge region, where important modifications are substitution at N10 with propargyl and methyl. Region **D** has been modified with the introduction of substituted benzene, thiophene, and the indol ring. Region **E** has also been modified for the introduction of different amino acids or of a benzene-ring substituent.

Many compounds need a complex metabolic pathway in order to increase their activity. They need a carrier called Reduced Folate Carrier (RFC) to enter the cell through the cell membrane. Then they can be functionalized as polyglutamates, being substrates of the enzyme folylpolyglutamyl synthethase (FPGS), which binds more glutamate residue to the first glutamic portion[67,68] (Fig. 7). The polyglutamic chain is important because owing to its hydrophilic chain the compound cannot reflux out of the cell. Its intracellular concentration is therefore high and its affinity for the TS enzyme is also higher, so its inhibition constant sometimes decreases as much as one hundred-fold. It seems that this step also increases the selectivity for the target tumor cells, because in some experiments in mice polyglutamation occurs faster than in the normal tissues.[69]

The multiple mechanism of resistance to the new selective TS inhibitors has been described elsewhere.[70] Of the most frequently studied compounds, ZD1694 (Tomudex) is RFC/FPGS dependent, and this metabolic pathway is responsible for the resistance observed. Resistance is reported as being due to decreased uptake via RFC, decreased FPGS activity (in turn due to lower enzymatic lev-

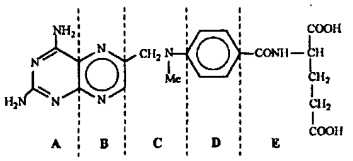
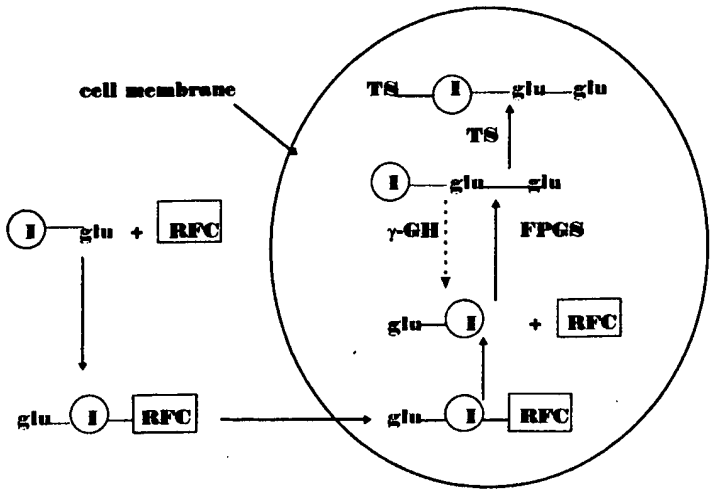


Figure 6. MTX divided in five regions that have been subjected to structure modifications.



I = Inhibitor, TS = Thymidylate Synthase, Glu = Glutamic residue, RFC = Reduced Folate Carrier

FPGS = Folylpolyglutamyl Synthetase, γ-GH = γ-Glutamyl Hydrolase

Figure 7. Metabolic pathway needed for antifolates inhibiting TS.

els or to mutated enzyme at the protein synthesis level) and elevation of intracellular TS levels by gene amplification.[71,72] These mechanisms are very similar to those observed in the case of FU resistance[73] and MTX resistance.[74–77] In FU resistance, gene modifications have been observed: A to G and T to C mutation occurs naturally while a Tyr33 to His33 mutation resulting in a decreased affinity of the 5-fluoro-2′-deoxy-5′-monophosphate for the mutated enzyme has also been reported.[78] Another mechanism of inhibition linked to FPGS activation has been observed, namely, increased levels of γ-glutamyl hydrolases (γ-GH). This is a family of enzymes in the mitochondria that selectively hydrolyze the γ-glutamyl bond of the polyglutamyl chain, thereby reducing the affinity of the inhibitors for the enzyme. This last type of resistance has been observed in some cases of prolonged treatment with MTX.[79,80]

Currently, there are three main directions in this field:

(a) antifolates structurally related to the folate cofactor and polyglutamation-dependent;
(b) antifolates structurally related to the folate cofactor, but polyglutamation-independent;
(c) folate inhibitors structurally unrelated to the folate cofactor, but with TS antifolate action and polyglutamation-independent.

The first class is the largest one, and the last class the smallest, having only recently been discovered. The most recent compounds have been synthesized by Agouron Pharmaceuticals in San Diego and Zeneca in England. Both companies followed similar research objectives in this field and have found new inhibitors with TS antifolate action that are under clinical trial. Agouron followed an approach based on the knowledge of the crystal structure (structure-based drug design) to find new inhibitors that are more potent and possess better pharmacokinetic properties. Zeneca has used a classical structure-activity approach to drug design.

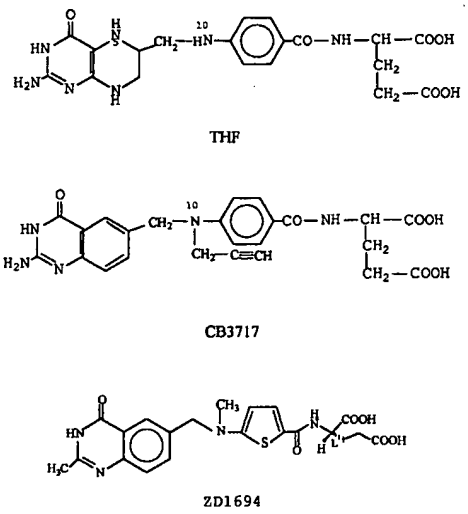


Figure 8. Selective antifolates (CB3717, ZD1694) structurally related to THF.

(a) The first TS inhibitor with antifolate action was MTX, which is historically known as a selective DHFR inhibitor. Its selectivity is limited to the type of DHFR inhibited (Human DHFR), but not to the target inside the cell, because it can also inhibit TS. Modifications of that structure gave the first selective TS antifolate inhibitor CB3717 (5,8-dideaza-N10-propargyl folic acid) (Fig. 8).

CB3717 was the first example of an antifolate selectively inhibiting TS with respect to DHFR. It was very important for the development of mechanistic studies[81] and of future inhibitors because it is very potent (14nm Ki) and it was possible to obtain the x-ray crystal structure of the ternary complex with EcTS and dUMP or with the antimetabolite FdUMP.[27] The x-ray crystal structures are reported below (Fig. 9).

CB3717 binds in the folate binding site, which is deep in the folate pocket, and it seems to be bound in an additional alternative binding site of the folate, usually occupied by the folate cofactor before starting the catalytic reaction.[82] Unfortunately, CB3717 is highly toxic to the kidney and liver, so the pharmacokinetic aspect needs improvements. Its metabolism is typical of the antifolates: it binds to the reduced folate carrier (RFC) and is polyglutamilated inside the cell by FPGS.

Hundreds of compounds have been developed and all of them have different modifications in each of the main domains of the folate general skeleton, but all remain close structural analogs of the cofactor.[83–96] In particular, the quinoline derivatives are still active as TS inhibitors and can be regarded as classical antifolates in the low micromolar and nanomolar range[83] (Fig. 10). Modification of the pyrazine ring into a furanic ring[65,97] decreases the TS inhibitory activity and favors DHFR inhibitory activity.

New inhibitors with folate or nonfolate structure[32,35,98–101] have been based on the x-ray crystal structure of the known ternary complexes of EcTS-dUMP-CB3717, EcTS-FdUMP-MTHF,[28] and EcTS-FdUMP-CB3717,[27] where the EcTS structure was used as a model for human TS because that was the only structure available in the past. The methodology consists of iterative cycles of design, synthesis, biological evaluation, and x-ray crystallography of the complexes of the new inhibitor with the target enzyme or a model of it.[99,102,103] The design consists of punctual modifications of the ligand chosen as a lead in order to establish, through suitable functional groups, specific bonds with the residues close to the inhibitor.

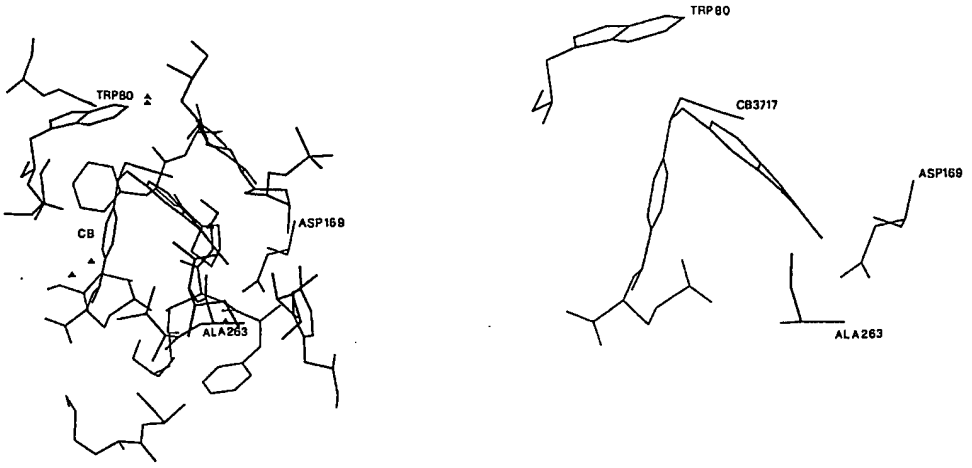
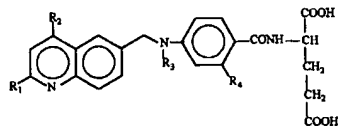


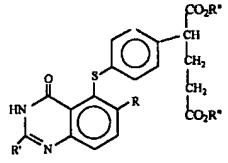
Figure 9. Details of the x-ray crystal structure EcTS-dUMP-CB3717. On the left the residues within 5 Å distance from the inhibitor are reported. On the right the inhibitor with three selected residues are reported.

Of the compounds recently designed with this method many inhibitors have been synthesized and some of them are actually in the advanced phase of clinical trialling (BW1839U43, ZD1694).[104–106] Other compounds of this class are some quinoline derivatives[83] and 5-(arylthio)-quinazolinones bearing the glutamate portion (IC50 = 42nM)[98,107] (Fig. 10).

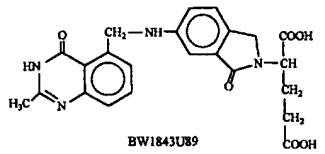
The latter ones have Ki around nanomolarity or lower. They are very lipophylic and pass the cell membrane passively but are still FPGS dependent. Some fluorine derivatives have also been syn-



quinoline derivatives



5-(arylthio)quinazolinone



BW1843U89

Figure 10. Quinoline derivatives, arylthio quinazolinones, and BW1843U89: antifolates inhibiting TS with FPGS-dependent metabolic pathway.

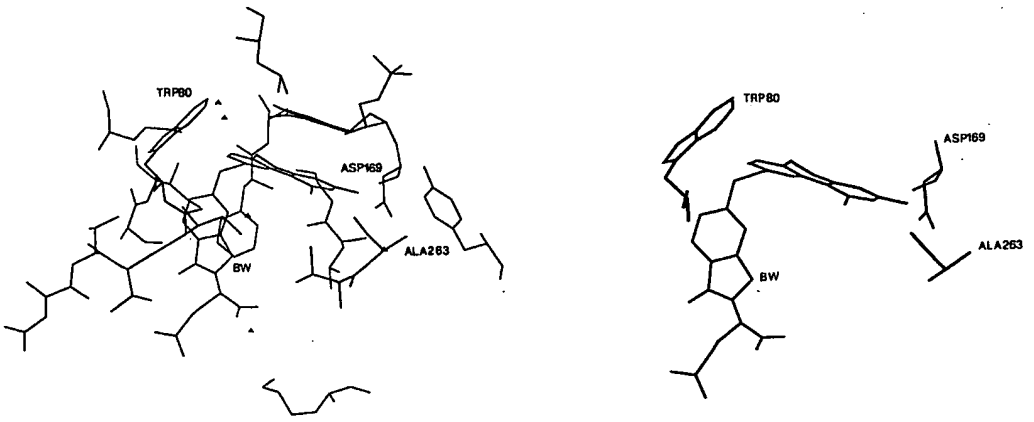


Figure 11. Details of the x-ray crystal structure of the ternary complex EcTS-dUMP-BW1843U89. On the left the residues within 5 Å distance from the inhibitor are reported. On the right BW183U89 and three selected residues are shown.

thesized[85] with no relevant improvements of the inhibitory potencies and of the pharmacokinetic quality.

A recent compound with a nonclassical antifolate structure is that synthesized by the Borroughs Wellcome research group: BW1843U89 (Fig. 10).[104,108,109]

It has a tricyclic aromatic ring, an indolyl-2-one ring that replaced the *p*-amino benzoyl group and has the glutamate moiety. It is a very active HTS inhibitor (Ki = 0.09nM) and uses RFC to pass the cell membrane, but does not require polyglutamation for activation. In fact, it is a substrate of FPGS, but the polyglutamated form has the same activity as the monoglutamated form.[109] The x-ray crystal structure of the ternary complex of the inhibitor with EcTS and dUMP and with EcTS and dGMP have been obtained[104,108] (Fig. 11).

It is worth noting that the binary complex displays two molecules of BW1843U89 per dimer of enzyme, while kinetic analysis can only account for one molecule per dimer. The two monomers from the crystal structure are not identical. The 24 h it takes to obtain the crystals allows the binding of two molecules instead of one; a conformational change occurs inside the crystal and both the monomers can bind a molecule of inhibitor. This is one of the first experiments to show the existence of a negative cooperativity between the two monomers, as already suspected from functional studies.[45,81,110]

(b) Modifications to the glutamate portion of the molecule cause it to avoid the FPGS substrate activity but not the use of RFC to pass the cell membrane.[111–115] Two series of compounds have been designed starting from the structure of IC198583, conserving the quinazoline structure.[113] It was observed that modifications to the γ-position of the glutamic acid residue in the dihydrofolate reductase inhibitor, MTX, resulted in compounds that are poor substrates or nonsubstrates for FPGS.[111] Following this example some compounds were synthesized in which the glutamic acid moiety was replaced with α-amino acids which could not be substrates for FPGS since they lack the γ-carboxylic acid group or lack completely the amino acid residue. TS inhibition resulted with the best one showing a 0.040 μM IC50 and being capable of inhibiting growth of L1210 at 0.09 μM IC50. If the amino acid chain is substituted with simple alkyl amide, benzyl or substituted benzyl heterocyclic benzylamide moieties they do not require FPGS and RFC[101] (Fig. 12). The best one inhibits HTS at 0.012 μM Ki and CCRF-CEM cell growth at 1.44 μM IC50. In general these compounds do not show very

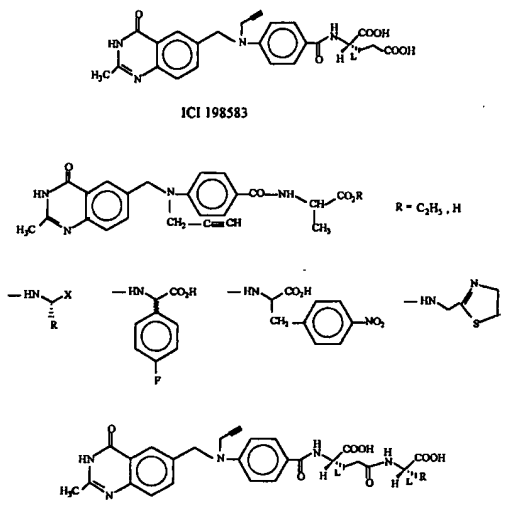


Figure 12. Antifolates TS inhibitors with altered glutamate moiety. Above ICI 198583, used as model compound, alkylamide derivatives and the γ-linked peptide derivatives are shown.

high potencies and show differences between the results on isolated enzyme and on cell growth indicating low target selectivity.

Other derivatives of ICI198583 modified at the aminoacid residue were synthesized with the aim to overcome the FPGS substrate activity but conserving the RFC transport system in order to offer some tumor selectivity[112,113]-γ-Linked, L-D, D-D, and D-L dipeptide analogues of ICI 198583 (2-desamino-2-methyl-N10-propargyl-5,8-dideazafolic acid) were synthesized. Compounds are better enzyme inhibitors than cell growth inhibitors (IC5011210 0.56 μM, 130 μM cell growth inhibition)[112] (Fig. 12).

A new series of liphophylic quinazoline inhibitors resembling the CB3717 structure, but lacking the glutamate residue have been synthesized to avoid the RFC/FPGS system. The results are promising, but also in this case the compounds are better enzyme inhibitors than cell culture inhibitors, thus indicating that the pharmacokinetic properties of the molecules have to be optimized[78,79,83,84,101] (Fig. 13).

(c)The non-analog antifolate inhibitors (NAAI) deserve special attention. These inhibitors were first described in 1991 in an article from Agouron.[32] This was the first published attempt to develop compounds with a skeleton completely different from that of the folate structure. They synthesized benz[c]indole derivatives, AG331 and AG (Fig. 13). The development of benz[c]indole derivatives[35] afforded AG331, as reported in 1994;[99] later[100,101] in 1996, a new class of extremely active compounds was patented[116] (Fig. 13).

Nonstructural analogy with the folate cofactor is the best way of identifying antifolate, folate-independent TS inhibitors. In this context can also be mentioned some phthalidic derivatives, whose lead was discovered in 1993 through analysis of the data bank of commercial compounds targeting a *de novo* design. Shoichet *et al.*[34] discovered phenolphthalein as a new lead following the docking method. They used DOCK as a computational method to evaluate the interaction between LcTS and

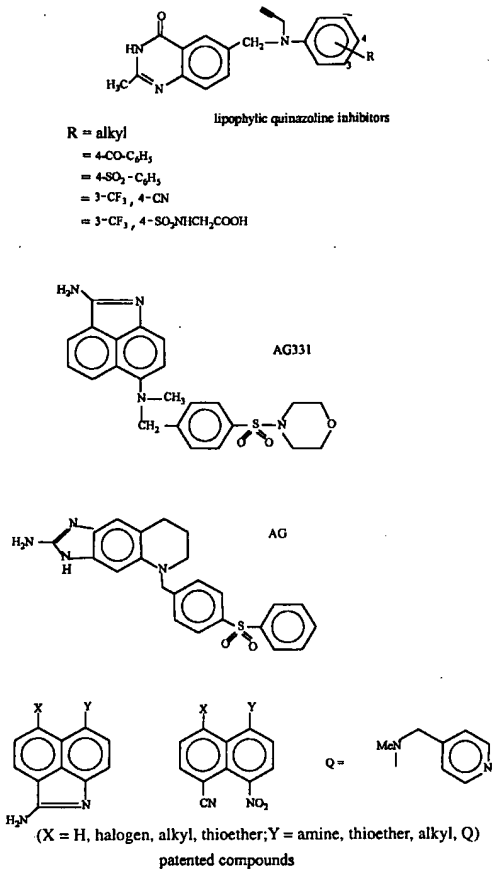
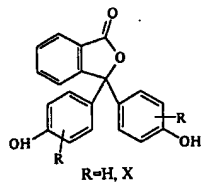


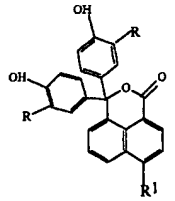
Figure 13. Lipophylic quinazoline derivatives and NAAI, AG331, and AG337, new patented compounds (RFC/FPGS independent).

many commercial compounds. Phenolphtalein was proposed as a new lead and from this substance new molecules have been designed and synthesized (Fig. 14).

Our research interest is in the field of the antifolate nonstructural analog of the folate targeting TS.[117–120] We applied a program of design, synthesis, crystallography, and biological assay of a new set of molecules with phthalidic and naphthalidic structures. Starting from the x-ray crystal structure of the binary complex of LcTS-phenolphtalein (Fig. 15) we applied a structure-based design and development. The compounds synthesized (Fig. 14) were tested against LcTS and proved to be low microMolar inhibitors. Accordingly, we tested them for species specificity against different TS species and found some interesting selective compounds versus HTS. Some naphthalidic derivatives were good antibacterial inhibitors, others were good antifungal inhibitors. We obtained the x-ray crystal structures of LcTS with two of these inhibitors: one selective and the second nonselective for LcTS rather than HTS. The two structures were markedly different in the binding site of the inhibitor. The nonselective compounds bind to the same area as phenolphthalein and the classical TS antifolates, while the selective compound binds to a new area of the folate binding pocket, 5 Å from the phenolphthalein binding site. It binds close to the small domain and to the carboxy terminal residues (His106, Val316), thus indicating that a topological selectivity can be the reason for the observed se-



Phenolphthalein



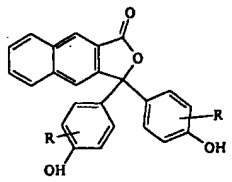


Figure 14. Recent NAAI, phthalidic derivatives.

lectivity. This observation is supported by the recent resolution of the x-ray crystal structure of HTS which is not very useful for purposes of drug design itself but can offer some suggestions. It has a eucariotic structure with some sequences specific for structure-function implications but lacking the small domain sequences (residues 90-139), thus suggesting that a molecule targeting the small domain could develop specific activity for the bacterial rather than for the human enzyme. Figure 16 shows the matching of the two x-ray crystal structures.

The naphthalidic derivatives can be regarded as leads of this new class of compounds that should be developed and modified in order to obtain more selective and more potent inhibitors. Many x-ray crystal structures of EcTS or LcTS enzyme complexed with some TS inhibitors have been obtained, but most of them are not available in the protein data bank. In particular, the x-ray structures of some non-analogs of folate with an indolyl structure have been obtained.[32,35,99] A brief description of the protein-inhibitor complex is given and it is understandable that the binding site of the inhibitors is

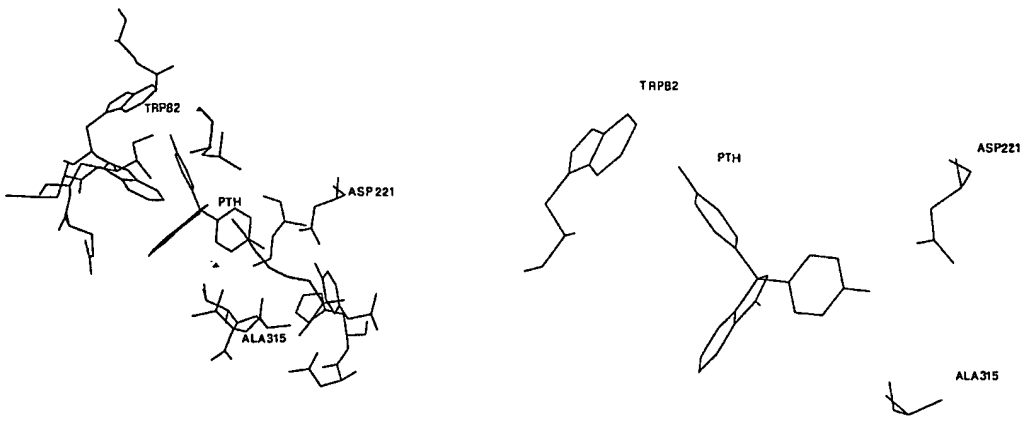


Figure 15. Detail of the LcTS-PTH binary complex with three selected residues are reported.

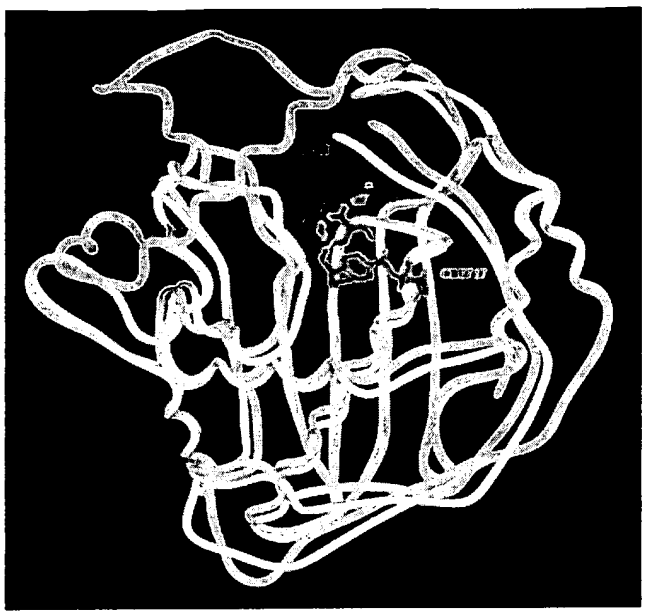


Figure 16. Rigid matching of the two x-ray crystal structures of the complexes EcTS-CB3717-dUMP (white structure) and LcTS-PTH (yellow structure). The small domain portion of the LcTS enzyme is clearly exceeding the EcTS structure.

always the same because the main residues are always involved in the binding and those of some importance are: Trp80, Asp169, Ala263, (EcTS numbering). These residues are the same as are involved in the binding of CB3717 and also phenolphthalein (Trp82, Asp221, Ala315, LcTS numbering). Figure 16 shows the matching of the EcTS-dUMP-CB3717 structure (white color) with the LcTS-phenolphthalein binary complex (yellow color). Owing to the absence of the small domain residues in EcTS, this part is clearly visible as a yellow extra chain at the top of the picture. EcTS and HTS both lack the small domain and have 40% structural homology, so the matching of the two structures leads to the same consideration. All the above mentioned compounds show the same biological activity profile: they are antifolates inhibiting HTS and also bacterial TS and this could be due to the binding of the compounds to the folate binding site.

The observations support our findings regarding the existence of alternative binding sites on the bacterial TS structure that are specific for antibacterial compounds.

V. CONCLUSIONS

Some authors have been wondering if TS inhibition research is at a dead end.[121] They question the recent results of TS-targeted chemotherapy, as in the case of the antifolate Tomudex (ZD1694) from Zeneca. Their objection applies in general to classical anticancer chemotherapy which targets enzymes involved in the key steps of nucleic acid synthesis. These enzymes are very often present in healthy human cells as well, so selectivity is difficult to achieve even in cases of known x-ray crystal structure, in which it would be possible to look directly at the 3-D structure and see if there are differences of which the researcher could take advantage; also, it is unusual to isolate and crystallize

human TS from healthy human cells. The structure of cloned HTS is solved but it is an unliganded structure with the catalytic cystein buried in the active site and so not suitable for structure-based studies. TS antifolates are very strong inhibitors but with low therapeutic indexes because of their high cytotoxicity, which is due to the low selectivity. We do not yet know the final results of the clinical studies on the latest antifolates from Agouron and Zeneca, but judging by the preliminary results they are less toxic. The structure-based research studies carried out at Agouron have been and continue to be successful.

A new aspect of TS inhibition is emerging from the advanced biological studies on TS. A self-regulatory function on its own promoter belonging to mRNA has already been assessed, but more regulatory functions are being proposed regarding the interaction with mRNA in other sites that apparently do not affect other synthetic path. Research in the field of TS is making progress thanks to new and valid tools; in particular, the species specificity studies recently embarked on afford new insight into the nature of TS. TS can be considered now a new target for antiproliferative drug design and similarly good selective antifolates can now be developed as they were in the case of DHFR.

This effort is dedicated to Rita Gollini, dear friend. I thank Prof. Daniel V. Santi and Piergiorgio Pecorari for the critical reading of the review, and Dr. Patrizia Verri for helping in the drawing of the manuscript.

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